

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis^{*[5]}

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Telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that inhibit several proinflammatory and Th1-biased immune responses. Synthetic oligodeoxynucleotides (ODN) expressing suppressive motifs can reproduce the down-regulatory activity of mammalian telomeric repeats and have proven effective in the prevention and treatment of several autoimmune and autoinflammatory diseases. Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation induced by LPS administration. Augmented expression of proinflammatory cytokines/chemokines such as TNF α , IL-6, and MCP1 and bactericidal nitric oxide production mediated by LPS contribute to the development of EIU. Suppressing these mediators using agents that are devoid of undesirable systemic side effects may help prevent the development of EIU. This study demonstrates the selective down-regulatory role of suppressive ODN after (i) local or (ii) systemic treatment in EIU-induced rabbits and mice. Our results indicate that suppressive ODN down-regulate at both the transcript and protein levels of several proinflammatory cytokines and chemokines as well as nitric oxide and co-stimulatory surface marker molecules when administered prior to, simultaneously with, or even after LPS challenge, thereby significantly reducing ocular inflammation in both rabbit and mouse eyes. These findings strongly suggest that suppressive ODN is a potent candidate for the prevention of uveitis and could be applied as a novel DNA-based immunoregulatory agent to control other autoimmune or autoinflammatory diseases.

DNA and RNA are the essential components of all living organisms. Accumulated evidence strongly suggests that these nucleic acids have multiple and complex effects on the immune system and are more than a blueprint of life (1, 2). On one hand, due to their high unmethylated CpG motif frequency, bacterial DNAs are recognized as “non-self” via TLR9 (Toll-like receptor 9) and trigger an innate immune response characterized by the proliferation and maturation of B cells, natural killer cells, and plasmacytoid dendritic cells and the secretion of T-helper

1-type cytokines, chemokines, and/or multivalent immunoglobulins (3–8). On the other hand, telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that can inhibit several TLR-dependent and TLR-independent Th1-mediated immune responses. Of note, these motifs are underrepresented in the prokaryotic genome. Synthetic single-stranded oligodeoxynucleotides (ODN)³ containing repetitive TTAGGG motifs mimic this effect (1, 9–11). Previous studies revealed that deleterious inflammatory responses to a host can be down-regulated by suppressive ODN. *In vitro*, suppressive ODN inhibits the production of several proinflammatory cytokines and chemokines induced by bacteria (1, 12–14). Furthermore, *in vivo* suppressive ODN administration reduces the frequency and severity of several autoimmune and inflammatory diseases such as arthritis, systemic lupus erythematosus, pulmonary inflammation, toxic shock, silicosis, and experimental autoimmune encephalomyelitis (10, 15–21).

Uveitis is an ophthalmic disorder that causes vision loss in developed countries (22, 23) and is characterized by acute, recurrent, or persistent ocular inflammation, the breakdown of the blood-ocular barrier, and infiltration of leukocytes (24). The underlying causes of uveitis can vary. For example, acute anterior uveitis is often associated with (i) Behcet disease, (ii) Reiter syndrome, and (iii) ankylosing spondylitis, as well as other systemic inflammatory diseases (25).

Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation. It is triggered by the administration of LPS, which is a component of the Gram-negative bacterial outer membrane (26). A ligand for TLR4, LPS enhances the expression of various proinflammatory cytokines and chemokines such as IL-6 (27, 28), TNF α (29), and MCP1 (monocyte chemoattractant protein 1) (30) and the production of nitric oxide. All of these mediators contribute to the breakdown of the blood-ocular barrier and infiltration of leukocytes, resulting in the development of EIU (26). It has been shown that suppressing proinflammatory cytokines, including IL-6, TNF α , MCP1, and inducible nitric-oxide synthase (iNOS), retards if not prevents the development of EIU (31). Conventional drugs used to control these concerted inflammatory activation are mainly immunosuppressive in character and are associated with undesirable systemic side effects (24). It is of the utmost importance to develop effective, less toxic agents that selec-

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³ The abbreviations used are: ODN, oligodeoxynucleotide(s); EIU, endotoxin-induced uveitis; iNOS, inducible nitric-oxide synthase.

tively block proinflammatory immune activation while eliminating the unwanted systemic side effects.

To date, the inhibitory effect of suppressive ODN on LPS-mediated EIU at both the local and systemic levels has not been studied by others. In this study, a very aggressive form of experimental uveitis was initiated via endotoxin administration. We investigated whether the suppressive ODN "A151" can inhibit the induction and development of ocular inflammation (before or at the time of LPS insult or even 2 h after LPS treatment) and help to reduce the symptoms of EIU in rabbits and mice. Our results revealed, for the first time, that A151 is capable of down-regulating the mRNA expression and protein levels of several potentially pathologic chemokines and cytokines at both the local and systemic levels. Consequently, suppressive ODN mimicking telomeric DNA offers a novel nucleic acid-based immunotherapeutic agent to control overexuberant undesirable immune responses such as seen in autoimmune and auto-inflammatory diseases.

EXPERIMENTAL PROCEDURES

Materials—All cell culture medium components were from HyClone. Cytokine pairs for ELISAs were from Endogen. LPS (isolated from *Escherichia coli*) was obtained from Sigma. The phosphorothioate-modified suppressive ODN A151 (24-mer, 5'-(TTAGGG)₄-3') and control ODN (24-mer, 5'-(TTACCC)₄-3') were obtained from Alpha DNA (Montreal, Canada). TRIzol G (AppliChem GmbH, Darmstadt, Germany) was used for RNA isolation. cDNAs were synthesized using a DyNAmoTM cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. DyNAzymeTM PCR Master Mix was used for PCRs.

Maintenance of Animals—Adult female BALB/c mice and adult New Zealand rabbits were used for the experiments. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled conditions at 22 °C with 12-h light and 12-h dark cycles. They were provided with unlimited access of food and water.

Induction of Endotoxin-induced Uveitis—Specific pathogen-free 10-week-old female BALB/c mice were injected intraperitoneally with 25, 50, 100, or 200 µg of LPS in 200 µl of PBS and/or suppressive ODN. Mice were killed at the end of clinical evaluation. Both eyes were enucleated and used for cytokine expression assays. Spleens were removed and split in two; splenocytes were incubated on tissue culture plates for 6, 12, and 24 h; and supernatants were collected for cytokine determination by ELISA. IL-6 was measured as an indicator of EIU response. The other half of the spleen was used to extract total RNA for further cytokine/chemokine gene transcript expression analysis by RT-PCR. In another experiment, rabbits (three to four animal/group, ~1500 g each; housed in the Ankara Hospital animal facility) were separated into different treatment groups, and EIU was initiated via intraocular LPS injection (100 µg) with or without suppressive ODN treatment. Eyes were removed, and further analyses as described for mice were conducted.

Clinical Evaluation and Histopathological Investigation—Animals were subjected to blind investigation by an ophthalmologist under a dissection microscope 18–24 h after injection, corresponding to the time of maximal severity of EIU. Clinical ocular inflammation was graded on a scale from 0 to 4 for each animal described previously (32): no sign of inflammation = 0; discrete inflammation in iris and conjunctiva = 1; dilatation of iris and conjunctiva vessels = 2; hyperemia in iris associated with Tyndall effect in anterior chamber = 3; in addition to the signs in scale 3, synechia or fibrin is formed = 4 (32). For histopathological investigations, enucleated eyes were fixed in 10% formalin for 24 h, washed with running tap water for 1 h, and placed in 60% ethyl alcohol for an extra 3 h. Eyes were embedded in paraffin, which was sectioned and stained with hematoxylin and eosin. Sections were examined blindly by a histopathologist, using score systems of severity ranging from 0 to 4. Focal non-granulomatous monocytic infiltration in the choroid, the ciliary body and retina were scored as 0.5. Retinal perivascular infiltration and monocytic infiltration in the vitreous were scored as 1. Granuloma formation in the uvea and retina and the presence of occluded retinal vasculitis along with photoreceptor folds, serous detachment, and loss of photoreceptor were scored as 2. In addition, the formation of Dalen-Fuchs nodules (granuloma at the level of the retinal pigmented epithelium) and the development of subretinal neovascularization were scored as 3 and 4 according to the number and size of the lesions (33).

Cytokine and IgM ELISAs and NO Assays—Immulon 2 HB microtiter plates (Thermo Scientific) were coated with anti-cytokine or anti-IgM antibodies (BD Pharmingen) and then blocked with PBS and 1% BSA (1, 34). Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine antibody followed by phosphatase-streptavidin (Perbio), whereas bound IgM was detected using phosphatase-conjugated anti-IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) as described (1). Nitric oxide detection by the Griess method was conducted on murine peritoneal exudate cells (10⁶/ml) after 12–36 h of *ex vivo* incubation as described by the supplier (Promega).

Analysis of Cell-surface Molecule Expression by FACS—2 × 10⁶ spleen cells/ml were isolated from 24-h post-treated mice. Cells were washed, fixed, and co-stained with one of the phycoerythrin-labeled anti-CD40, anti-CD86, and anti-ICAM-1 and FITC-labeled cell-specific antibodies (*i.e.* CD11c for dendritic cells, CD11b for macrophages, and B220 for B cells (BD Pharmingen)) for 30 min at room temperature. Following washing, they were studied using a FACSCalibur (BD Biosciences) and analyzed with CellQuest Pro software.

Cytokine and Chemokine RT-PCR—Animals were injected with LPS and/or suppressive ODN. Total RNA was extracted from the eyes or spleens of the mice 4–6 h later (or from the irises or corneas of the rabbits), reverse-transcribed, and amplified to obtain cDNA in a standard PCR for 30 cycles using primers for mouse- or rabbit-specific target genes (Table 1) as described previously (1, 34). PCR-amplified material was separated on 1.5% agarose gels and visualized under UV light after ethidium bromide staining.

TABLE 1

Oligonucleotide PCR primers used in mouse or rabbit experiments

m, mouse; rb, rabbit.

Primer	Forward	Reverse	Product
m β -actin ^a	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA	bp
mIP10 ^a	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT	450
miNOS ^a	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	127
mMIP1 α ^b	ACCATGACACTCTGCAACCA	AGGCATTTCAGTTCCAGGTCA	95
mIL-5 ^a	AGCACAGTGGTGAAGAGACCTT	TCCAATGCATAGCTGGTGATT	238
mIL-15 ^a	CATCCATCTCGTCTACTTGTGT	CATCTATCCAGTTGGCCTCTGT	117
mIL-18 ^b	GATCAAAGTGCCAGTGAACC	ACAAACCCCTCCACCTAAC	126
mMCP1 ^b	AGGTCCTGTCATGCTTCTG	TCTGGACCCATTCTTCTTG	384
mMIP3 α ^b	CGTCTGCTCTTCTTGTCTTT	CCTTTTCACCCAGTTCTGCT	249
mCXCL16 ^b	CCTTGTCTCTTGCCTTCTTC	GGTTGGGTGTGCTCTTGTGT	250
mMIP1 β ^b	CCAGCTCTGTGCAACCTAA	CTGTCTGCCTCTTTTGGTCA	384
rbGAPDH ^c	TCACCATCTTCCAGGAGCGA	CACAATGCCGAAGTGGTCGT	250
rbIL-6 ^c	GCTCCTGGTGGTGGCTAC	GGGTGGCTTCTTCATTCAA	319
rbIL-1 β ^c	GCCGATGGTCCAATTACAT	ACAAGACCTGCCGAAGCT	450
			121

^a Taken from Ref. 43.^b In house-designed primers.^c Taken from Ref. 44.

Statistical Analysis—Assays were performed in triplicate on at least three to five different cell preparations. Statistical significance between untreated (or control) and treated groups was evaluated using Student's *t* test.

RESULTS

EIU is an established animal model of acute ocular inflammation. It is induced by either systemic or intravitreal administration of LPS, the major component of Gram-negative bacteria. LPS acts through the TLR4-triggering proinflammatory signaling cascade. The expression of Th1 cytokines and chemokines, including IL-6, IL-1 β , and MIP3 α (macrophage inflammatory protein 3 α), contributes to the development of EIU.

This study was performed with 82 mice and 26 rabbits. Initial experiments were conducted to optimize the induction of EIU (supplement Fig. 1). For the mouse experiments, systemic administration of LPS doses between 25 and 100 μ g/mouse were sufficient to induce uveitic eyes within 24 h as judged by clinical and histopathological investigations (supplement Figs. 1 and 2). For the rabbit experiments, intraocular 100- μ g LPS injection was found to be optimal to induce EIU. Following local or intraperitoneal LPS and/or suppressive ODN administration, rabbit and mouse eyes were removed, and RNAs from the irises, vitreous, and corneas of the rabbit eyes were obtained. PCR was run with the cDNA from each sample, and the mRNA levels of IL-6, IL-15, IP10 (interferon- γ -inducible protein 10), iNOS, MIP1 α , IL-18, MIP3 α , CXCL16 (CXC chemokine ligand 16), MIP1 β , and IL-1 β were monitored. In addition, 24 h post-LPS and/or A151 treatment, splenocyte suspensions were incubated *ex vivo* for 6–24 h, and IgM, IL-6, IL-10, IL-12, and IFN γ levels from the supernatants were determined by ELISA. FACS analyses were conducted on spleen cells to monitor co-stimulatory/surface marker molecule expressions.

The results indicated that in rabbits, suppressive ODN administered before or after 100- μ g LPS treatment or co-injected with LPS significantly down-regulated the expression of IL-1 β message from the iris (Fig. 1A). In cornea, IL-6 was down-regulated when suppressive ODN was given before or simultaneously with LPS administration (Fig. 1B). There was no sig-

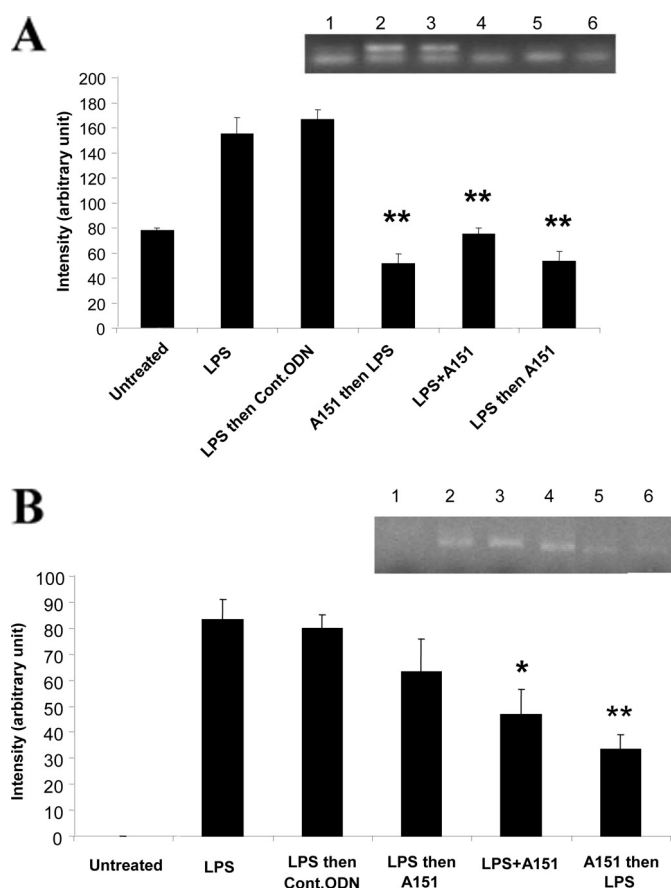


FIGURE 1. Suppressive A151 ODN administration after LPS challenge significantly down-regulates IL-1 β and IL-6 expression in the iris and cornea, respectively. Rabbits were injected intraocularly with 100 μ g of LPS and 250 μ g of suppressive ODN. The average of densitometric measurements of four animals for IL-1 β mRNA from iris (A) and IL-6 mRNA from cornea (B) is shown. Insets are the representative gel image of each group labeled from untreated to A151 and then LPS as 1 to 6. *, $p < 0.05$; **, $p < 0.01$ between LPS-treated and A151 ODN-co-administered groups.

nificant inhibitory effect when A151 was given post-LPS treatment. In all these experiments, the mRNA message reduction was suppressive ODN-dependent because control ODN administration did not show any benefit for alleviation of LPS reactivity.

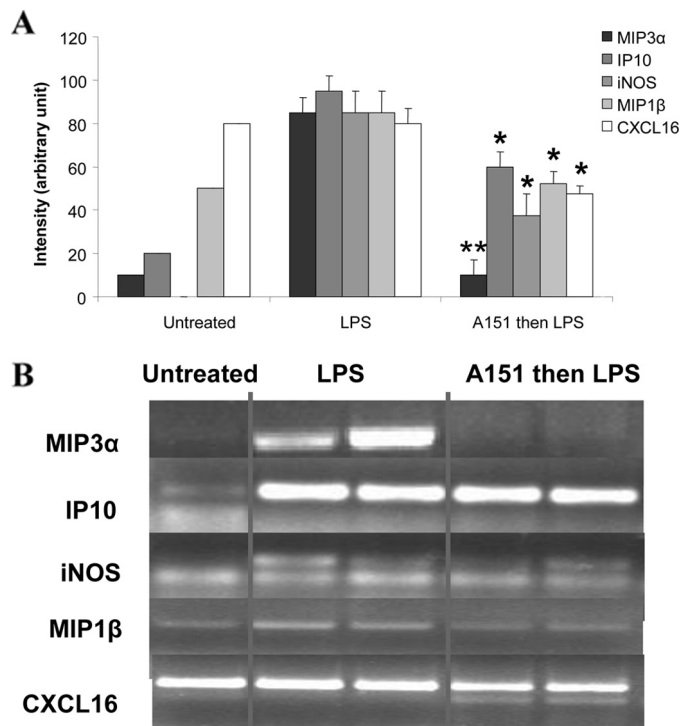


FIGURE 2. A, suppressive A151 ODN treatment after 100- μ g LPS challenge significantly down-regulates IP10, MIP3 α , iNOS, MIP1 β , and CXCL16 expression levels in the eyes of mice. Mice were injected intraperitoneally with 100 μ g of LPS and 250 μ g of suppressive ODN and killed 18 h after injection. *, $p < 0.05$; **, $p < 0.01$ between LPS-treated and LPS + A151 ODN-co-administered groups. B, representative gel image.

In the murine EIU model, mice tolerated up to 100- μ g LPS intraperitoneal treatment. Doses >150 μ g caused animals to succumb to endotoxin treatment. The mouse experiments were conducted with three doses of LPS: 25, 50, and 100 μ g. Suppressive A151 ODN and control ODN (2 h before and at the time of LPS injection and 2 h after LPS treatment) were used in the range of 100–250 μ g. Although in rabbits, the injection of ODN and endotoxin was intraocular, in mice, injections were given intraperitoneally in 200 μ l of PBS.

The results showed that when 250 μ g of suppressive ODN was administered before LPS injection (2 h), it significantly down-regulated the expression of IP10, iNOS, MIP3 α , CXCL16, and MIP1 β in the 100- μ g LPS-injected mouse EIU model (Figs. 2, A and B). Other cytokines such as MIP1 α and IL-18 also showed substantial but insignificant down-regulation at these doses (data not shown). To understand the systemic effect of suppressive A151, IL-6 secreted from murine splenocytes after *ex vivo* incubation for up to 24 h in culture was monitored by ELISA (Fig. 3). Our results revealed that suppressive ODN was able to reduce $>65\%$ of the secreted IL-6 (430 ± 70 and 135 ± 55 ng/ml for LPS and A151 + LPS groups, respectively). Co-administration of suppressive ODN with LPS significantly decreased cytokine mRNA levels *in vivo* or cytokine production in *ex vivo* spleen cells ($p < 0.01$) (Figs. 2 and 3). These effects were attributable to the activity of suppressive motifs because control ODN did not reduce the cytokine production elicited by co-administered LPS (Figs. 1–3).

The (TTAGGG)₄ multimers inhibited LPS-dependent up-regulation of co-stimulatory and surface marker molecules on

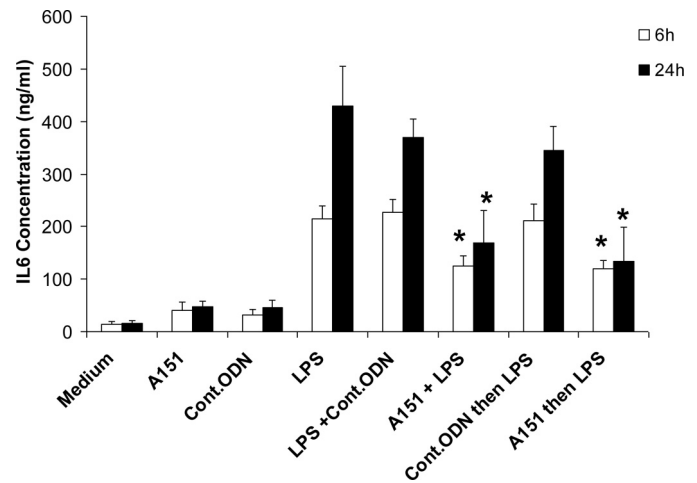


FIGURE 3. **Suppressive A151 ODN administration significantly suppresses IL-6 release from murine splenocytes.** Mice were injected intraperitoneally with 100 μ g of LPS and 250 μ g of suppressive or control ODN as further indicated. Spleen cells were removed (24 h post injection) and incubated 6–24 h, and supernatants were collected for cytokine ELISA. IL-6 was measured as an indicator of EIU response. *, $p < 0.05$, between LPS and LPS + A151 groups.

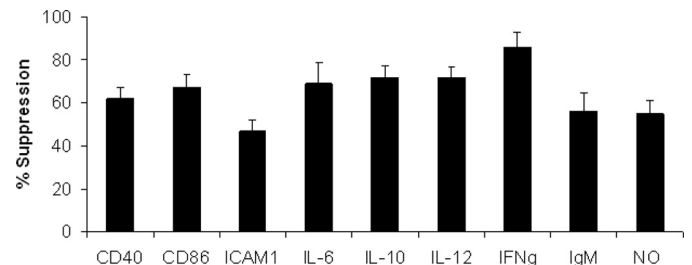


FIGURE 4. **Inhibitory effect of suppressive ODN on LPS-mediated immune activation.** The levels of CD40, CD86, and ICAM-1 expression (mean fluorescence intensity) were determined by FACS 24 h after *in vivo* injection of LPS or LPS plus A151 (50 μ g of LPS and 250 μ g of ODN). IL-6, IL-10, IL-12, IFN γ , and IgM levels in culture supernatants were determined by ELISA following 36 h of *ex vivo* incubation. The Griess method was used to detect nitric oxide in isolated peritoneal exudate cells supernatants 24 h post-treatment. % Suppression was calculated by the following formula: $(1 - ((\text{activation by LPS} + \text{suppressive ODN}) - (\text{background}) / (\text{activation by LPS} + \text{control ODN}) - (\text{background}))) \times 100$.

antigen-presenting cells (CD40, CD86, and ICAM-1), IgM production by B cells, and NO release from peritoneal macrophages ($p < 0.01$) (Fig. 4). Furthermore, co-administration of LPS (50 μ g) with A151 ODN (250 μ g) inhibited $>65\%$ of several immunoregulatory and inflammatory cytokines (*i.e.* IL-6, IL-10, and IL-12; $p < 0.001$) (Fig. 4). This reduction reached $>85\%$ for IFN γ (176 ± 29 and 26 ± 15 ng/ml for LPS and A151 + LPS groups, respectively).

DISCUSSION

In this study, we examined the effect of synthetic telomeric repeat units (suppressive A151 ODN) localized at the end of mammalian chromosomes on EIU, which is an established animal model of acute ocular inflammation in both mouse and rabbit models. The results indicated that suppressive ODN was able to down-regulate the expression and protein levels of several proinflammatory and immunoregulatory cytokines/chemokines at local and systemic levels when administered (i) prior to, (ii) simultaneously with, or (iii) even after LPS challenge (Figs. 2–4).

Compared with local injection of (intraocular) LPS and/or LPS- and control ODN-treated rabbits, suppressive ODN-administrated animals exhibited reduced levels of IL-1 β and IL-6 expression in the iris and cornea, respectively. In the mouse model, the results revealed that pretreatment with 250 μ g of suppressive ODN reduced the expression of IP10, iNOS, MIP1 α , IL-18, MIP3 α , CXCL16, and MIP1 β in 100- μ g LPS-injected mice. In another experiment, with different doses of suppressive ODN and LPS, suppressive ODN also down-regulated the expression of MCP1, which is an important chemokine for monocyte chemoattraction (data not shown). The suppressive action of this class of ODN was not only on the mRNA levels of several Th1-type cytokines and chemokines but also on the secreted protein level. ELISA experiments showed that suppressive ODN pre- and post-treatments significantly diminished IL-6 secretion at 6 and 24 h; simultaneous administration of suppressive ODN also reduced IL-6 production.

Several studies indicated that suppressing proinflammatory cytokines, including IL-6, TNF α , MCP1, and iNOS, prevents the development of EIU (26, 29, 30, 31). Here, we have shown that either local (intraocular) or systemic (intraperitoneal) administration of suppressive A151 ODN can significantly reduce several proinflammatory cytokines and chemokines even 2 h after *in vivo* LPS challenge.

Corticosteroids and chemotherapeutic agents are currently in use in uveitis therapy (35). However, long-term treatment with these drugs may have grave side effects such as increased intraocular pressure (36) and cytotoxicity (37) and thus limit their use (25, 33, 35). Therefore, a new therapeutic strategy is urgently needed (38, 39). The mechanism of action of this novel ODN-based immunosuppressive drug candidate is currently unknown. Previous studies revealed that suppressive ODN can inhibit immune response by blocking the stimulatory effects of CpG motifs (1, 15). It also has been shown by Shirota *et al.* (10) that suppressive A151 ODN can also protect mice from lethal endotoxic shock that is induced by LPS. It has been shown that suppressive ODN can also inhibit several signal transduction cascades related to the production of Th1 cytokines such as IFN γ and IL-12 by binding and inhibiting the phosphorylation of STAT1 (signal transducer and activator of transcription 1) and STAT4 proteins (10, 13). Our study has demonstrated that suppressive ODN can block immune responses mediated by endotoxin in the eye (an immune privileged site), an established animal model of acute ocular inflammation. Recently, Fujimoto *et al.* (40) reported that suppressive A151 ODN can inhibit ocular inflammation in two murine models, IRBP (interphotoreceptor retinoid-binding protein)-induced experimental autoimmune uveitis and adoptively transferred ocular inflammation. These forms are antigen-driven and, compared with LPS, are significantly less aggressive forms of experimental uveitis models. The control of LPS-mediated EIU at both the local and systemic levels has not been studied by others and increases the breadth of the suppressive ODN-mediated therapy for the eye. Collectively, these observations support the provocative possibility that the evolutionary expansion of TTAGGG repeats in telomeres, in addition to known properties such as, protecting genomic DNA from degradation, and chromosome capping (41, 42) may also be linked to their ability

to down-regulate sustained/pathologic microbe-associated molecular pattern-induced immunity. In conclusion, we have provided evidence that suppressive A151 ODN is able to significantly reduce the ocular inflammatory responses in both rabbit and murine EIU models.

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